

QUANTITATIVE MEASUREMENT OF THE FORMATION AND DEGRADATION
OF NEUROTRANSMITTER MONOAMINES AT THE BLOOD-BRAIN
INTERPHASE IN VARIOUS SPECIES INCLUDING MAN

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INITIATIVE MEASUREMENT OF THE FORMATION AND DEGRADATION
OF NOVEL STIMULANT MONOMERIS AT THE DOUBT-RAIN
INITIATION: VARIOUS SELECTS INCLUDING MAIN

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ABSTRACT

The activities of tyrosine hydroxylase, aromatic L-aminoacid decarboxylase, monoamine oxidase and catechol-O-methyltransferase were determined in microvessels (capillaries and venules), parenchymal arterioles and pial vessels from rat brains, and the decarboxylase activity was compared in brain microvessels from rabbit, cat, dog, pig, cow, baboon, and man. Cranial sympathectomy was performed to estimate the neuronal contribution to the enzyme activities. All vascular regions had substantial activities of the various enzymes studied. The activity of aromatic L-aminoacid decarboxylase in cerebral microvessels was high in rat, dog, pig, cow and man; intermediary in rabbit and cat; and low in baboon. In addition to this enzyme, cerebral microvessels also contained tyrosine hydroxylase and monoamine oxidase. Aromatic aminoacid decarboxylase and monoamine oxidase serve an enzymatic barrier function at the microvascular level, whereas the main function of tyrosine hydroxylase is probably to synthesize monoamines within nerve terminals that are in close association with microvessels, under the conditions used for preparation of the microvascular fraction. In larger intracerebral and pial vessels monoamine oxidase was present both in the wall itself and in perivascular sympathetic nerves; the remaining two enzymes had primarily a neuronal localization. The latter types of vessels also had catechol-O-methyltransferase in their wall proper.

ABSTRACT

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The structural unity of the endothelial lining at the level of the blood-brain barrier (REESE & KARNOVSKY, 1967) prevents to a great extent - although not totally (OLDENDORF, 1971; HARDEBO et al., 1977b) - the passage of polar and water-soluble substances, like neurotransmitter monoamines, into the brain parenchyma. However, by aid of a saturable transport system the monoamine precursors, such as L-3,4-dihydroxyphenylalanine (L-DOPA) are extracted from the circulation into the endothelial cells and pericytes of brain microvessels (WADE & KATZMAN, 1975). L-DOPA (BERTLER et al., 1966; OWMAN & ROSENGREN, 1967; BARTHO-LINI et al., 1971) and also L-5hydroxytryptophan (L-5-HTP) (BERTLER et al., 1964) and dihydroxyphenylserine (CONSTANTINIDIS et al., 1975) are then decarboxylated within the microvessel walls. Thus, the presence of aromatic L-amino acid decarboxylase (AAD) in the microvessel wall constitutes an enzymatic barrier (BERTLER et al., 1966), impeding the passage of these amino acids into the brain. This barrier mechanism, as characterized in the mouse and rat, is unique for brain parenchymal microvessels, and has not been detected in vessels of peripheral tissues or in brain parenchymal arterioles and pial arteries and arterioles (OWMAN & ROSENGREN, 1967; HARDEBO et al., 1979a). The precursor L-tyrosine is also highly extracted from the brain circulation (OLDENDORF, 1971; OLDENDORF, 1973; PARDRIDGE, 1977). The amines formed are locally deaminated by monoamine oxidase (MAO) within the brain microvessel wall (BERTLER et al., 1966; SPECTOR et al., 1977; LAI & SPECTOR, 1978; HARDEBO et al., 1977a, 1979a). Also peripheral vessels are capable of degrading monoamines as reflected by the presence of MAO and catechol-O-methyl transferase (COMT) in the smooth muscle cell layer (AXELROD et al., 1959; VERITY et al., 1972; LEVIN & WILSON, 1977).

It can be assumed that the enzymatic blood-brain barrier is highly important in regulating the entrance of monoamine precursors into the brain parenchyma in accordance with the physiological demand, and in the prevention of circulating monoamines to enter the brain, thus maintaining an adequate neurotransmission function in the neuron systems. The capacity of the enzymatic barrier was elucidated in the present study by quantitative measurement of TOH, AAD, MAO and COMT in the walls of isolated pial and brain parenchymal vessels, with particular emphasis on the presence of microvascular AAD in various species including man.

METHODS

Animals. The study was performed on vessels from adult animals: 161 male Sprague-Dawley rats, 8 albino rabbits, 3 cats, 4 dogs, 3 pigs, 3 cows, and 4 baboons. Macroscopically intact brain tissue from 6 humans was obtained during extensive resections in conjunction with tumor surgery. In order to differentiate between neuronal and extraneuronal enzyme activity the perivascular sympathetic nerve plexus was eliminated by bilateral removal of the superior cervical ganglion 10 days prior to the measurement in 45 of the rats. The laboratory animals had free access to food and water. They were killed under light ether or nembutal anesthesia by perfusion through the left ventricle of the heart with 0.9% saline in order to eliminate any error in the determinations caused by the presence of blood.

Preparation of pial vessels and cerebral microvessel fractions. After perfusion and decapitation of the laboratory animals, the brains were immediately removed and kept in ice-cold 67 mM phosphate buffer (pH 7.4) throughout the procedure. Brains from pig and cow were obtained from a nearby slaughterhouse and were dissected out within 15 min after death. Human brain tissue (frontal, temporal or cerebellar cortex with underlying white matter) was immediately placed in ice-cold buffer and transported to the laboratory within 30 minutes after its removal. The meninges, including the pial membrane and its vessels, were carefully torn off, and the choroid plexuses were removed. The proximal part of the anterior and middle cerebral arteries and its branches (or the local pial vessels in the human cerebrocortical tissue) were collected for determination of enzyme activity. White matter was dissected away as far as possible.

The essentially grey matter of the parenchyma thus remaining was handled as follows: Tissue pooled from 4-6 rats, 2-3 rabbits or one cat brain, as well as tissue from various brain regions from pig, cow, dog, baboon or man was chopped with a razor blade, and then further disrupted by some ten strokes up and down through three different 10-20 ml plastic syringes equipped with a nylon net (pore size 1000 μ , 500 μ and 280 μ , respectively) glued to their open cut end (Fig. 1). The material was subsequently gently homogenized by hand by a relatively loosely fitting Teflon pestle in a smooth glass tube (0.1 mm clearance). The homogenate was washed through a nylon sieve with 225 μ pore size. The material remaining on the sieve, after extensive washing, was

re-homogenized and re-sieved 3-4 times through the same, carefully washed sieve. This repeated and careful homogenization will disrupt most of the remaining clusters of neurons and glia. Previous light-microscopic analysis has shown that the material finally remaining of the sieve consists of large parenchymal vessels, and clusters of large vessels branching off into numerous microvessels (Fig. 2b); most capillaries and venules pass this sieve. The fraction is inevitably contaminated by a few small clusters of neurons and glia. The tissue passing this sieve was collected and sieved through another sieve with 75 μ pore size. The material remaining on this sieve, after extensive washing, re-homogenization and re-sieving, consists of microvessels (capillaries and venules) and a few larger vessels (Fig. 2a). This fraction is purely vascular, contaminated only with a few glial endfeet (HARDEBO *et al.*, 1977a) and possibly perivascular nerves. The yield of microvessels is less than 1/1000 of the original wet weight of grey matter. Individual neurons, glia, small microvessel segments and subcellular fragments pass the 75 μ sieve. BRENDDEL *et al.* (1974) have shown that a mixed fraction of intracerebral vessels, prepared under similar conditions, is metabolically active.

Estimation of enzyme activity. The two different fractions (2-3 mg wet weight) of parenchymal vessels from essentially grey matter, and the pial vessels as well as homogenized tissue from the whole brain of 4 rats were re-homogenized in 20-60 μ l 10 mM potassium phosphate buffer (pH 6.5), and aliquots were removed for radiometric estimation of TOH activity according to the method of HENDRY & IVERSEN (1971), and AAD and MAO as described by FONNUM (1976). COMT activity was estimated according to the method described by AXELROD (1962). Each enzymatic assay was linear with respect to protein concentration and incubation time. A further series of estimations of AAD activity was undertaken in microvascular fractions from the various species studied, by incubation in the presence of L-DOPA under anoxic conditions (BERTLER & ROSENGREN, 1959), and the amount of dopamine formed was determined according to HÄGGENDAL (1963).

Tissue protein was determined according to the method of LOWRY *et al.* (1951).

Statistics. Differences between mean values were analyzed with Student's t-test for unpaired data.

RESULTS

Determinations of TOH was performed on material from rat, where activity

was found in all vascular preparations. The activity was considerably lower than in whole brain tissue. Some of the TOH activity remained in pial vessels after cervical sympathectomy (Table 1). This should be compared with rabbit pial vessel in which practically no activity is left following such denervation (HARDEBO *et al.*, 1977a). Sympathectomy did not affect the TOH activity in the microvascular fraction.

The AAD activity was determined in various species and, in some animals, also in different regions (cortex, caudate nucleus and cerebellum); the findings are illustrated in Fig. 3. A substantial activity, well above blanks, was found in all animals studied, although species variations were noticed. The AAD activity in the caudate nucleus and cortex was somewhat higher than in the cerebellum. Sympathetic denervation, carried out in the rat, reduced the AAD activity in the brain microvessels by 20-30 per cent, whereas only little enzyme activity remained in the preparations of pial arteries (Table 1).

Both the pial and large parenchymal vessels showed MAO activity (Table 1), part of it remaining even after sympathectomy. Also in the brain microvessel fraction - mainly consisting of endothelial cells and pericytes - a considerable MAO activity was found. The pial and large parenchymal vessels also contained COMT (Table 1). The activity of this enzyme was unchanged after cervical sympathectomy. Essentially no activity was found in the microvessel fraction (of non-denervated animals).

DISCUSSION

Normal functions of the brain rely upon an adequate control of the level of neurotransmitters, *e.g.* monoamines and their precursors, in the brain extracellular fluid compartment. Accordingly, the entrance of these substances from the circulation must be strictly regulated, which is accomplished by the blood-brain barrier. One aspect of this barrier is the presence of monoamine-related enzymes at the blood-brain interface.

The brain vascular fractions prepared by the disruption-filtration procedure has a high degree of purity and reproducibility with regard to the different types of vessels contained in the various fractions. The fraction of large CNS vessels consists of arteries, arterioles and also microvessels; the separate microvessel fraction contains capillaries and venules with insignificant addition of larger vessels. The former fraction

is inevitably contaminated by small clusters of glia and neurons, and glial endfeet have been shown to adhere to the wall of some of the microvessels in the latter (HARDEBO et al., 1977a). Since all parts of the cerebrovascular bed is well supplied with adrenergic nerves, it can be expected that perivascular terminals will also be present in the different fractions: the brain capillaries are innervated by fibres that may be of intracerebral origin (RENNELS & NELSON, 1975; SWANSON et al., 1977), whereas other parenchymal vessels are supplied with sympathetic nerves arising from the superior cervical ganglia (EDVINSSON & OWMAN, 1977). This implies that with the technique used in the present study, another aspect of the monoamine-related enzymes in the brain vessels is the inevitable contamination by perivascular adrenergic nerve terminals.

Tyrosine hydroxylase. The amino acid L-tyrosine - the main precursor for the neurotransmitter catecholamines - is present in the circulation in high concentrations (e.g. AMBROSE et al., 1969) and is to a high extent extracted from the brain circulation (OLDENDORF, 1971, 1973; PARDRIDGE, 1977). The cerebral microvessels constitute the major location for the passage across the blood-brain interphase, where tyrosine shares an uptake site for neutral amino acids in common with phenylalanine, methionine, histidine, cysteine, valine, isoleucine, leucine, tryptophan, threonine and DOPA (CHIRIGOS et al., 1969, OLDENDORF, 1973, PARDRIDGE, 1977). TOH activity resistant to removal of the adrenergic nerve supply (see also HARDEBO et al., 1977a) was present in the wall of brain microvessels. No reduction in microvascular TOH activity occurs after combined treatment with sympathectomy and 6-hydroxydopamine (HARDEBO et al., 1977a). This finding reflects the purity of the fraction with regard to vessels: a contamination with parenchymal catecholamine neurons would have caused a further decrease in TOH activity after the 6-hydroxydopamine treatment. This finding does not, however, rule out the possibility of a neuronal location of the microvascular TOH activity, since vascular adrenergic innervation is comparatively resistant to 6-hydroxydopamine treatment (BERKOWITZ et al., 1972).

There is reason to believe that the TOH activity has an intracellular localization since washing in 0.4M phosphate buffer during the filtration procedure does not affect the absolute values of the activity (HARDEBO et al., 1977a). This indicates that no contamination of membrane-bound enzyme (cf. FONNUM, 1970) obtained during the disruption - filtration process has occurred.

It is tempting to postulate that the discrepancy between the high availability of L-tyrosine and the considerable cellular uptake at the blood-brain interphase on one hand, and the regulated central demand of the amino acid on the other, might indicate that the presence of TOH in brain microvessels serves a (bidirectional) barrier function for tyrosine. However, the amino acid transport mechanism across the blood-brain interphase is probably the main limiting factor in determining the availability of this amino acid to the brain (see PARDRIDGE & OLDENDORF, 1977). There is so far no evidence that this presence of TOH reflects extraneuronal synthesis of catecholamine stores; it rather functions to synthesize catecholamines within (non-sympathetic) nerve terminals that are located in close association with the blood vessels.

Aromatic L-amino acid decarboxylase. The presence of an enzymatic barrier to aromatic amino acids, such as DOPA and 5-HTP, operating through decarboxylation to the corresponding monoamine, which is thereby trapped in the microvessel wall, was first shown by BERTLER et al. (1966) in the mouse and rat. The presence of decarboxylase activity in rat intracerebral vessels is confirmed in the present study by direct measurements on vascular preparations, and agrees with observations on the fate of radiolabelled L-DOPA at the blood-brain interphase (WADE & KATZMAN, 1975). The activity in baboon and also in the macaque (LANGELIER et al., 1972) microvessels was considerably lower than in the other species studied. Rabbit and cat also displayed a relatively low microvascular activity, in agreement with findings upon administration of L-DOPA in vivo (DOWSON & LASZLO, 1971; LANGELIER et al., 1972; HARDEBO et al., 1979b) and after in vitro incubation of brain slices in the presence of L-DOPA (DOWSON, 1973; HARDEBO et al., 1979c). A slight regional difference was found in decarboxylase activity: the activity in the microvessels of caudate nucleus and cortex was higher than in those of cerebellum. This confirms findings in the rat in which regional differences in the decarboxylating capacity was studied under in vivo conditions (HARDEBO et al., 1979b). There is evidence that the decarboxylase barrier has an upper limit beyond which unchanged L-DOPA leaks further into the brain parenchyma (HARDEBO et al., 1977c).

The sympathetic innervation of pial vessels (NIELSEN & OWMAN, 1967) as well as intracerebral arterioles (EDVINSSON & OWMAN, 1977) is reflected in the finding that most of the AAD activity was eliminated following sympathectomy. However, the level of AAD activity in the microvascular fraction was only slightly diminished by sympathectomy (see also HARDEBO et al., 1977a). Also in the choroid plexus a

considerable AAD activity is found, both in neuronal and extraneuronal structures (LINDVALL *et al.*, 1979).

Not until recently has it been possible to detect any $\underline{\underline{L}}$ -DOPA in the systemic circulation. The normal concentration is probably low (about 3×10^{-8} M; HANSSON *et al.*, 1978) but may vary with the dietary intake, even if $\underline{\underline{L}}$ -DOPA is decarboxylated to a high content in various peripheral tissues. 5-HTP has still not been detected in the circulation (e.g. MAGNUSSEN & HOLBAEK, 1978). If circulating $\underline{\underline{L}}$ -DOPA were not metabolized within the brain microvessel wall, it would be taken up across the blood-brain barrier by the neutral amino acid carrier system. Only after administration of high amounts of $\underline{\underline{L}}$ -DOPA in the medical treatment of Parkinson's disease are considerable amounts of circulating $\underline{\underline{L}}$ -DOPA found, particularly during combined treatment with a decarboxylase inhibitor. The AAD activity in the brain vessel walls may also be involved in a more complex enzymatic barrier mechanism for other circulating amino acids such as phenylalanine, tyrosine or tryptophan or may function as a link in a hitherto unknown metabolic pathway across the blood-brain interphase.

Monoamine oxidase and catechol-O-methyl transferase. Due to the existence of a structural blood-brain barrier, represented by the closed tight junctions between the endothelial cells and the paucity of micropinocytotic vesicles in these cells (REESE KARNOVSKY, 1967), only a minor amount (about 3-5%) of neurotransmitter monoamines pass into the brain, probably uniformly in various regions of the parenchyma (OLDENDORF, 1971; HARDEBO *et al.*, 1977b; HARDEBO & NILSSON, 1979). However, as pointed out above, monoamines may accumulate in the microvessel walls after the uptake of the precursor amino acid followed by local synthesis. Once formed, the amine is rapidly metabolized by MAO which is also present in the microvessel walls. Pre-requisites thus exist for another aspect of the enzymatic barrier mechanism at the level of brain vessels, namely, the finding of MAO activity in the microvessel fraction, and MAO and COMT activity in pial vessels (including the choroid plexus; LINDVALL *et al.*, 1979) and parenchymal arterioles (see also LAI & SPECTOR, 1978).

Within the wall per se of vessels such as arteries, arterioles and veins these enzymes are probably located in the smooth muscle cell layer (VERITY *et al.*, 1972; LEVIN & WILSON, 1977). In addition, MAO is also located in the perivascular sympathetic nerves. It is usually found that vascular MAO of neuronal origin is primarily of the A type, whereas MAO in the smooth muscle cell layer is primarily of type B (LEVIN &

WILSON, 1977). In isolated brain vessels (mixture of large and small vessels) the presence of both MAO A and B has been reported, even after removal of perivascular sympathetic nerves (LAI & SPECTOR, 1978). Hence, 5-hydroxytryptamine, adrenaline and noradrenaline (substrates primarily for type A) and dopamine (substrate to a varying degree for both type A and B) (HOUSLAY *et al.*, 1976; YODIM, 1977) may be deaminated within the walls of brain vessels. The primary action of these enzymes in arteries and arterioles may be related to the local inactivation of amines that have a vasomotor function, reaching the smooth muscle media from the perivascular nerves or from the circulation. In this latter respect, the smooth muscle uptake (GILLESPIE, 1973; BUCHAN *et al.*, 1974) and breakdown by MAO and COMT of amines that have reached beyond the endothelial lining also may represent a kind of barrier mechanism. This is probably particularly important in the central nervous system, where the passage across the endothelium is low and the subsequent degradation therefore efficient. That dopamine indeed can reach the brain vascular smooth muscle cells and induce a transient vasoconstriction (before the amine is metabolized), is consistent with the finding of a short-lasting reduction in CBF upon intracarotid injection in the rat of a bolus of noradrenaline (HARDEBO *et al.*, 1979d). However, due to the minimal passage across the endothelial barrier, the response is smaller than would be expected from a comparison with sympathomimetic effects on the peripheral vascular bed (see also review by EDVINSSON & MACKENZIE, 1976).

Microvascular MAO, providing an enzymatic breakdown of the amine trapped within the endothelial cells and pericytes, should not only be considered as a gate mechanism for the entrance of circulating neurotransmitters into the brain parenchyma, but also as an inactivation mechanism for excess of neurotransmitter present in the brain extracellular compartment since monoamines are actively taken up into the wall of microvessels from the abluminal side (HAMBERGER, 1967; HARDEBO *et al.*, 1979a; HARDEBO & OWMAN, 1979). The possibility should also be considered that the endothelium with its amine enzymes may not only serve a simple barrier function: the amine uptake and enzyme mechanisms may play a role in the function of the endothelial cells themselves, which may be target structures for circulating and neurogenically released amines in *e.g.*, contractile processes (OWMAN *et al.*, 1977). In this context it should be recalled that a direct innervation of the endothelial cells and pericytes has recently been demonstrated by electron microscopy (RENNELS & NELSON, 1975; SWANSON *et al.*, 1977).

One aspect of the functional implication of the presence of MAO and COMT at

the blood-brain interphase has been studied in measurements of amine-induced changes in the cerebral blood flow and metabolism. The response to the circulating amines can be modified when MAO or COMT is inhibited (MACKENZIE et al., 1975; MCCALDEN & EIDELMAN, 1976; MCCULLOCH & HARPER, 1978; HARDEBO et al., 1979d) or when the blood-brain barrier is transiently opened by e.g. a hypertonic insult (MACKENZIE et al., 1976; EDVINSSON et al., 1978; HARPER & MACKENZIE, 1977) or circumvented by intraventricular administration of the amines (MACKENZIE et al., 1976).

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Table 1. Activities of the enzymes tyrosine hydroxylase (TOH), aromatic L-aminoacid decarboxylase (AAD), monoamine oxidase (MAO), and catechol-O-methyltransferase (COMT) in the whole brain and in pial, large CNS and small CNS vessels of untreated and sympathectomized (SyX) rat. SyX was performed by bilateral removal of the superior cervical ganglion 10 days prior to determinations. Appropriate blanks subtracted.

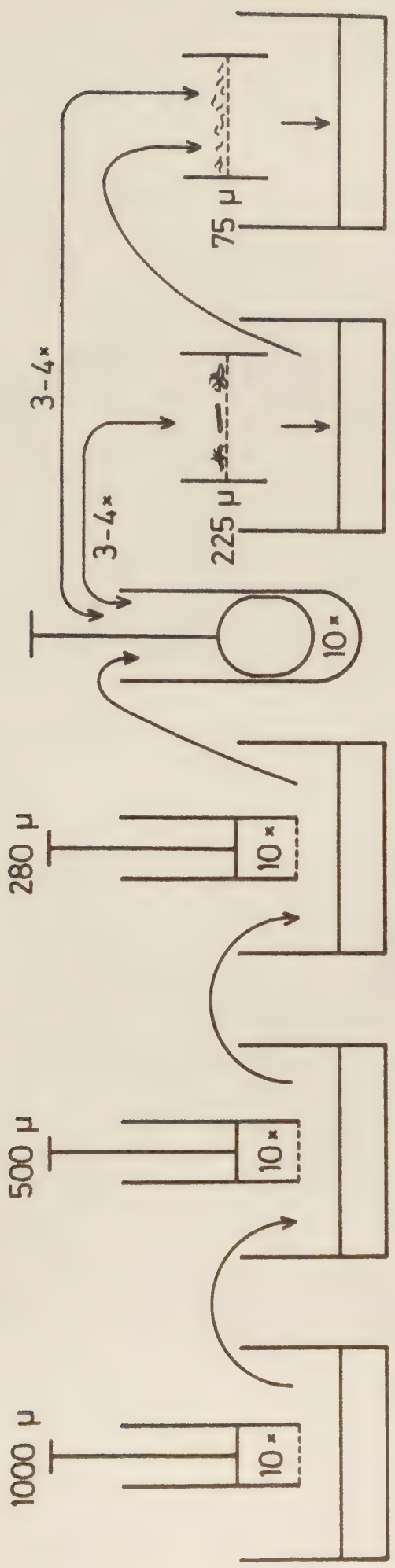
Tissue analyzed	TOH pM/mg prot/h	AAD nM/mg prot/h	MAO nM/mg prot/h	COMT pM/mg prot/h
Whole brain	86.50 ± 3.27 (4)	5.07 ± 0.09 (4)	20.40 ± 1.32 (4)	720 (1)
Pial vessels untreated	13.53 ± 1.27 (4)	2.68 ± 0.25 (6)	19.34 ± 1.58 (3)	531 ± 80 (3)
SyX	8.32 ± 1.07 (3)*	0.35 ± 0.12 (4)***	8.14 ± 0.43 (4)**	520 (1)
Large CNS vessels untreated	19.11 ± 3.61 (3)	1.88 ± 0.17 (3)	3.38 ± 0.42 (3)	143 ± 48 (5)
SyX	not detectable	not detectable	2.03 ± 0.24 (3)*	
Small CNS vessels untreated	17.80 ± 2.07 (3)	4.85 ± 1.02 (4)	5.32 ± 0.49 (8)	25 ± 17 (3)
SyX	19.22 ± 2.95 (3)n.s.	3.40 ± 0.60 (4)n.s.	4.64 ± 0.42 (4)n.s.	

Mean values ± S.E.M. Number of determinations within parenthesis. Comparison of intact vs denervated tissue according to Student's t-test: *p < 0.05, **0.001 < p < 0.01, ***p < 0.001, n.s. = not significant.

Fig. 1. Principles for obtaining the various brain vascular fractions. For details, see text.

Fig. 2. (a) Representative illustration of a microvessel fraction from man, temporal cortex, methylene blue staining. The nuclei of the endothelial cells and pericytes appear black. Blood corpuscles are seen in the lumen. X 135. (b) A cluster of microvessels originating from an arteriole, illustrating representative material obtained on the 225 μ sieve. Rat brain. X 80.

Fig. 3. Aromatic L-aminoacid decarboxylase (AAD) activity, expressed as mM dopamine formed per mg tissue protein per hr, in microvessel fractions obtained from the grey matter of brain tissue from various species. In rat, rabbit, and cat whole brains were used for determinations, whereas in dog, pig, cow, baboon, and man selected regions were used (cerebellar cortex, caudate nucleus and cerebral cortex). The activity in baboon microvessels is very low, and the activity in rabbit and cat are also lower than in the other species studied. A considerable activity is found in man. Mean values + S.E.M., number of determinations within parenthesis.



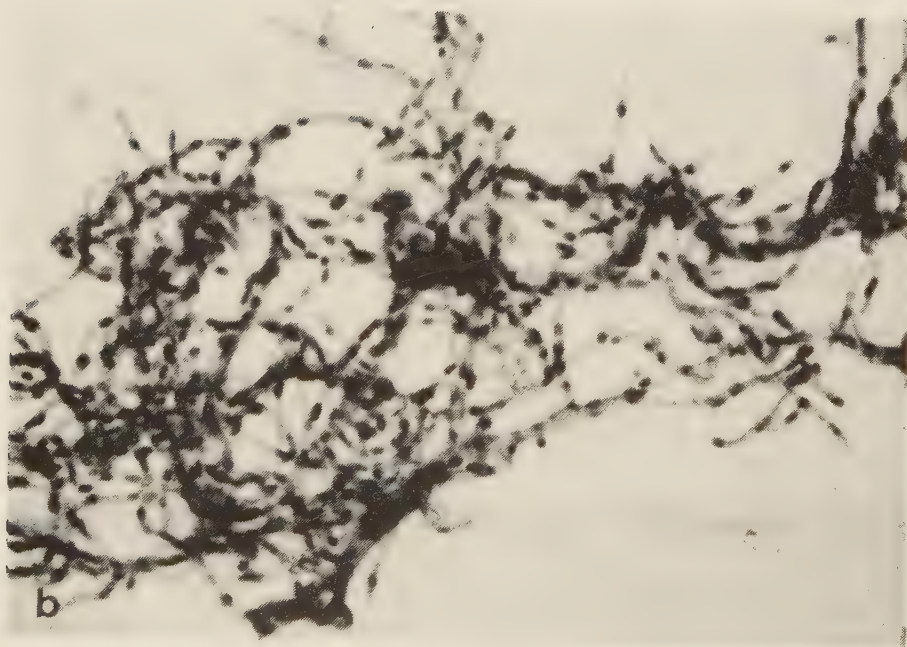
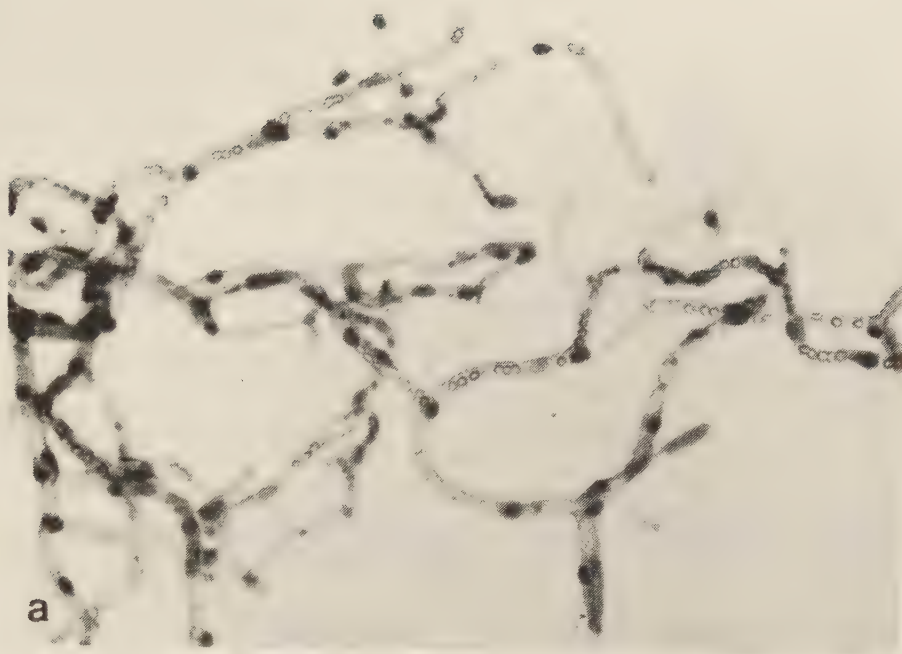
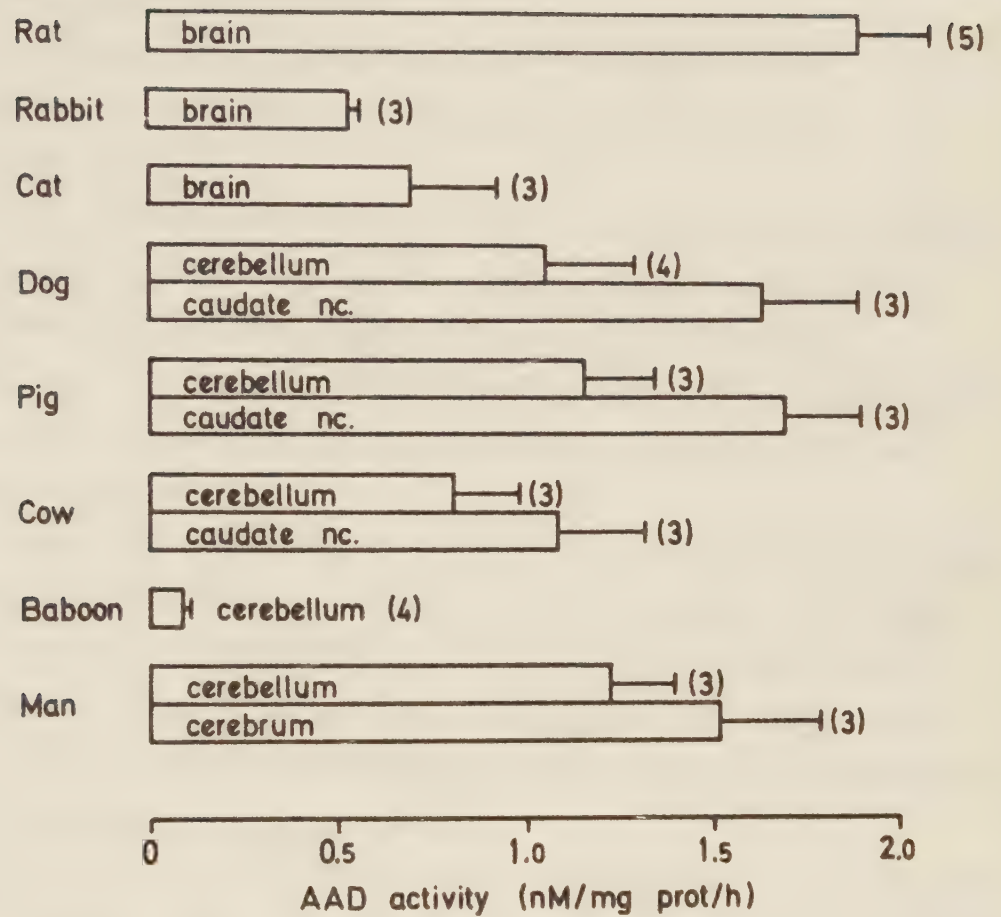


Fig. 2

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